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Bidirectional effects of the neuroactive steroid tetrahydrodeoxycorticosterone on GABA-activated Cl^- currents in cultured rat hypothalamic neurons

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1 The non-genomic effects of tetrahydrodeoxycorticosterone (THDOC; 5-alpha-pregnane-3-alpha, 21-diol-20-one) were studied in cultured hypothalamic neurons of the rat.

2 The effects of THDOC (10 nM – 1 μM) on responses to different concentrations of exogenously applied GABA and on spontaneous inhibitory postsynaptic currents (IPSCs) were measured with whole-cell voltage clamp recordings.

3 Application of GABA induced inward currents with dose-dependently increasing amplitudes (up to 3.9 nA at a holding potential of -20 mV). High doses of THDOC (100 nM – 1 μM) induced small inward currents on its own (14 ± 3 and 24 ± 3 pA, respectively).

4 Simultaneous application of 10 μM GABA with 100 nM or 1 μM THDOC increased current amplitudes by 125 and 128%, respectively. At 10 nM THDOC exerted no consistent effects on GABA currents.

5 Responses to 1 μM of GABA were modulated in a bidirectional manner by different doses of THDOC: 10 nM THDOC reduced the amplitude of GABA responses to 80% ($P = 0.018$, $n = 15$), whereas 100 nM and 1 μM THDOC enhanced the GABA response to 115 and 180% ($P = 0.0007$, $n = 15$), respectively.

6 The time constant of decay of spontaneous inhibitory postsynaptic currents (IPSCs) was reversibly increased from 91 ± 10 to 314 ± 34 ms ($n = 3$) by the application of THDOC (1 μM). The amplitudes of the IPSCs were not affected by THDOC.

7 These data indicate that THDOC modulates GABA responses of hypothalamic neurons in a bidirectional manner, resulting in a complex tuning of neuronal excitability in the hypothalamus.

Keywords: GABA currents; THDOC; neuroactive steroids; hypothalamic neurons

Abbreviations: ACSF, artificial cerebrospinal fluid; DMEM, Dulbecco's modified Eagle medium; FCS, foetal calf serum; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid; HPA, hypothalamus-pituitary-adrenocortex-axis; IPSC, inhibitory postsynaptic current; MEM, minimum essential medium; PS, pregenolone sulphate; THDOC, 5-alpha-pregnane-3-alpha, 21-diol-20-one; THP, tetrahydroprogesterone

Introduction

Two distinct cellular effects have been described for neuroactive steroids in the nervous system, including both genomic and non-genomic mechanisms. The endogenous neuroactive steroid 5α-pregnane-3α, 21-diol, 20-one (tetrahydrodeoxycorticosterone, THDOC) was shown to activate the progesterone receptor in COS-1 cells following intracellular oxidation of THDOC to 5 α-pregnane-21-ol-3,20-dione (Rupprecht *et al*., 1993), or through direct membrane actions *via* modulation of $GABA_A$ receptor-mediated Cl^- currents.

The direct interaction of neuroactive steroids with the $GABA_A$ receptor has been shown for different preparations using various methods (for review see Lambert *et al*., 1995). Neuroactive steroids modulate benzodiazepine binding as well as the binding of GABA and muscimol in the brain or to fractions of neuronal membranes (Majewska *et al*., 1986; Majewska, 1990; Harrison *et al*., 1987a; Peters *et al*., 1988; Turner *et al*., 1989; Goodnough & Hawkinson, 1995; Nguyen *et al*., 1995). Binding of the convulsant *t*-butylbicyclophosphorothionate (TBPS) to the $GABA_A$ receptor (Majewska *et al*., 1986; Gee *et al*., 1988; Turner *et al*., 1990) and the uptake of radioactive labelled Cl^- into synaptoneurosomes was also shown to be modulated by these neuroactive steroids (Majewska *et al*., 1986; Morrow *et al*., 1989; Purdy *et al*., 1990). Finally, using electrophysiological methods, it was demonstrated that A-ring reduced pregnanes modulate GABA-induced Cl^- currents in cultured rat hippocampal and spinal cord neurons (Majewska *et al*., 1986; Barker *et al*., 1987; Harrison *et al*., 1987b; Twyman & MacDonald, 1992; Hauser *et al*., 1995), in slice preparations of the rat cortex (Teschemacher *et al*., 1995), and in the rat mesencephalic reticular formation *in situ* (Ermirio *et al*., 1989).

The THDOC concentrations in the brain and plasma are very low in nonstressed male rats (< 1 ng g^{-1} or ng ml^{-1}, respectively). After stress or during pathophysiological conditions THDOC concentrations increase 4–20 fold in plasma and to 10–20 nM in the brain (Purdy *et al*., 1991). The elevation of the THDOC concentration in the brain follows the swim-stress-induced elevation of the plasma-level of deoxycorticosterone with some delay (Purdy *et al*., 1991). This finding suggests that THDOC is synthesized in the brain from peripheral deoxycorticosterone, which increases

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in response to stress *via* the blood brain barrier also in the brain. Steroid hormones may modulate the excitability of hypothalamic neurons by genomic and non-genomic mechanisms, which in turn might be involved in controlling the activity of the hypothalamus-pituitary-adrenocortex-(HPA-) axis.

In the present study we focus on the non-genomic actions of the naturally occurring neuroactive steroid THDOC on the $GABA_A$ receptor-mediated Cl^- currents in cultured hypothalamic neurons of the rat.

Methods

Tissue blocks containing the hypothalamic region were removed from foetal rats (Wistar) at day 17 of gestation (E17) and collected in ice-cold Dulbecco's modified Eagle medium (DMEM, GIBCO, Eggenstein, Germany), containing 25 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES). Proteolytic digestion at 37°C with 0.1% Trypsin/Versen (BIOCHROM, Berlin, Germany) was stopped after 15 min by adding minimum essential medium (MEM, GIBCO, Eggenstein, Germany) containing 10% foetal calf serum (FCS, GIBCO, Eggenstein, Germany). The tissue was dissociated into single cells by gentle trituration through fire-polished pasteur pipettes. The cells were plated in MEM/10% FCS on poly-L-lysine coated glass coverslips in 24-well culture dishes (NUNC, Life Technologies, Eggenstein, Germany). The initial density was 2×10^5 living cells per cm^2. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After 24 h the medium was changed to a defined serum-free medium (modified TNB100, Vedder *et al*., 1993), unless otherwise stated. After 3 days *in vitro* (DIV3) the medium was changed every 2.5 days. The cells were used for electrophysiological recordings after DIV6.

Neurons were recorded in the whole-cell voltage-clamp configuration of the giga-seal technique (Hamill *et al*., 1981) under visual control using an inverted microscope (IM35, ZEISS, Oberkochen, Germany). The culture was kept in an artificial cerebrospinal fluid (ACSF), containing (in mM): NaCl 150, KCl 2.5, NaH_2PO_4 1.25, $CaCl_2$ 2, $MgSO_4$ 2, glucose 10, HEPES 10, sucrose 20. The pH was adjusted to 7.4 with HCl/NaOH. Patch pipettes (resistance about 4 MΩ) were pulled from borosilicate glass (Nr.: 1403005, HILGENBERG, Malsfeld, Germany) using a horizontal pipette puller (ZEITZ-Instruments, Augsburg, Germany) and were filled with a solution containing (in mM): KCl 130, NaCl 15, $MgCl_2$ 2, $CaCl_2$ 1, EGTA 11, HEPES 10, ATP 2, 0.25 cyclic AMP (pH 7.25). THDOC was dissolved in ethanol and diluted with ACSF to the desired concentration. The solvent concentration was below 0.01% (20 μM – 2 mM). All substances were purchased from SIGMA (Deisenhofen, Germany) or MERCK (Darmstadt, Germany). Steroids and GABA were applied locally in various concentrations *via* a multibarrel/singletip superfusion device (modified from Boll & Lux, 1985). Twelve separate solenoid-valve gated channels were assembled to a single glass pipette with a tip of 200 μm in diameter. The orifice of the application pipette was aimed at the recorded cell, adherent to the bottom of the recording chamber. The recorded cell was continously superfused, with either GABA-free or GABA-containing solution for control experiments (flow rate 200 $\mu l\ min^{-1}$). A 5 s GABA-pulse was delivered every 60 s. To study the modulatory potency of steroids, they were added to the GABA containing solutions at the indicated concentrations.

Current signals were recorded at a holding potential of −70 mV, unless otherwise stated, with a single electrode voltage/current clamp amplifier (NPI, Tamm, Germany). The data were digitized and stored on a LSI 11-73 computer with the data-acquisition software INTESV. The data were analysed off-line with the AUTESP software, kindly provided by H. Zucker, MPI of Psychiatry. All values are expressed as the arithmetic mean ± standard error of the mean (s.e.mean). Statistical analysis was performed using commercial software, and the significance of the effects was tested applying the Wilcoxon signed-rank test.

Results

Properties of cultured hypothalamic neurons

Immediately after disrupting the membrane patch, the zero current potential was −68 ± 2 mV ($n = 35$) and the neuronal input resistance was 510 ± 116 MΩ. Only cells in which action potentials (AP) or sodium inward currents could be elicited were regarded as neurons and used for the experiments. The AP amplitudes averaged 70 ± 4 mV ($n = 11$). The number of neurons which exhibited spontaneous synaptic currents was higher in high density cultures. Superfusion of the culture with 50 μM bicuculline methiodide greatly reduced the amplitude of these inwardly directed postsynaptic currents or blocked them totally, indicating that these postsynaptic currents were mainly due to the activity of GABAergic neurons converging onto the recorded neuron.

The membrane potential, input resistance and spike amplitude of the cells selected for analysis are within the range known for hypothalamic neurons in slice preparations (Wuarin & Dudek, 1993) and neurons of the rat hypothalamus grown in cell culture (Misgeld & Swandulla, 1989; Swandulla & Misgeld, 1990; Müller *et al*., 1992).

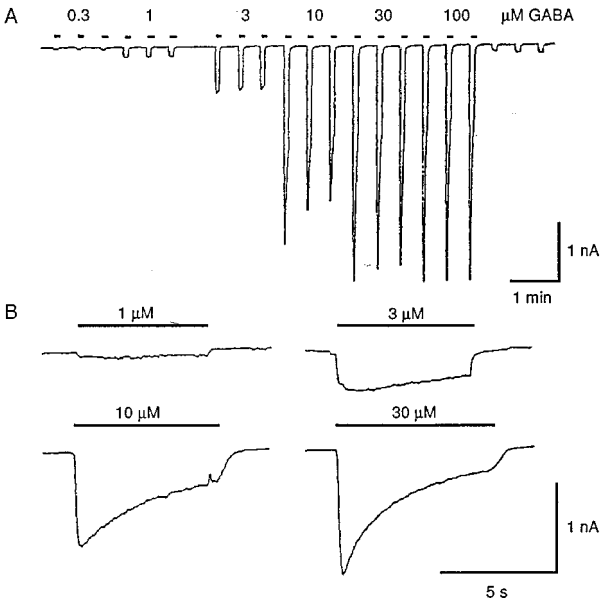

Figure 1 GABA responses of cultured rat hypothalamic neurons. (A) Application of 0.3 – 100 μM GABA induced inward currents of increasing amplitude. Small currents, elicited by 0.3 or 1 μM GABA (≤300 pA) reached a plateau and remained constant during the application. Higher concentrations of GABA induced larger currents (>300 pA) which developed a maximum and decayed to a plateau. (B) Records of the neuron in (A), shown with another time scale to display the kinetics of the responses.

Properties of GABA-induced currents

Application of GABA induced inward currents in all neurons investigated. The amplitudes of the GABA-induced currents depended on the concentration of GABA and the holding potential. Stepwise shifts of the holding potential to less negative values decreased the amplitudes of the GABA-induced currents. The currents reversed polarity at 2.7 ± 1.8 mV ($n=3$), close to the reversal potential for Cl^- set by the symmetrical Cl^- solutions (solutions were nominally free of bicarbonate). GABA responses were virtually abolished by 50 μM bicuculline methiodide (four of four cells), i.e. they were mediated by $GABA_A$ receptors. The amplitudes of the induced currents increased dose-dependently (Figure 1). In some neurons even concentrations of GABA as low as 0.3 μM induced sizeable inward currents ($\geqslant 50$ pA). The currents induced by 0.3–1 μM GABA ($\leqslant 200$ pA) slowly increased and then remained constant during the application. Higher concentrations of GABA (>3 μM) induced larger currents ($\geqslant 300$ pA) which developed faster and decayed to a plateau. Application of GABA (100 μM) induced in some neurons inward currents which saturated the amplifier. In these cases the holding potential was decreased to -20 mV. From the maximal peak amplitude of 3.9 nA at the holding potential of -20 mV a membrane conductance of up to 193 nS (67 ± 24 nS) was estimated. The dose-response curve for the averaged current amplitudes ($n=9$) is shown in Figure 2.

The time constant of decay of GABA-induced currents was a function of the amplitude of the peak current and the GABA concentration. The time constant of decay ranged from 2.7–6.9 s with 3 μM GABA (4.7 ± 0.6 s), and from 0.75 s to 2.6 s with 100 μM GABA (1.46 ± 0.07 s). The rise time to peak ranged from 2.3 s with 1 μM GABA (0.68 ± 0.17 s) to 90 ms with 100 μM GABA (0.16 ± 0.02 s).

Modulation of GABA responses by THDOC

To test for possible direct effects, THDOC was applied by itself in the two higher concentrations. At 100 nM, THDOC induced small inward currents (14 ± 3 pA) in five of 19 neurons (26%), and at 1 μM, THDOC elicited a small response of 24 ± 3 pA in 15 of 26 (58%) neurons. In each of the remaining neurons the effects were below the resolution governed by the noise. THDOC alone caused only marginal effects by itself, but invariably enhanced GABA-induced responses. To obtain a more complete view of the interaction, we investigated the effects of three concentrations of THDOC on the responses to two intermediate concentrations of GABA (1 and 10 μM). THDOC (10 nM) had no consistent effect on the membrane currents induced by 10 μM GABA ($n=9$; see Figure 4). In the neuron illustrated in Figure 3, the currents induced by 10 μM GABA (587 pA) were augmented to 647 and 717 pA by 100 nM and 1 μM THDOC, respectively, (each value represents

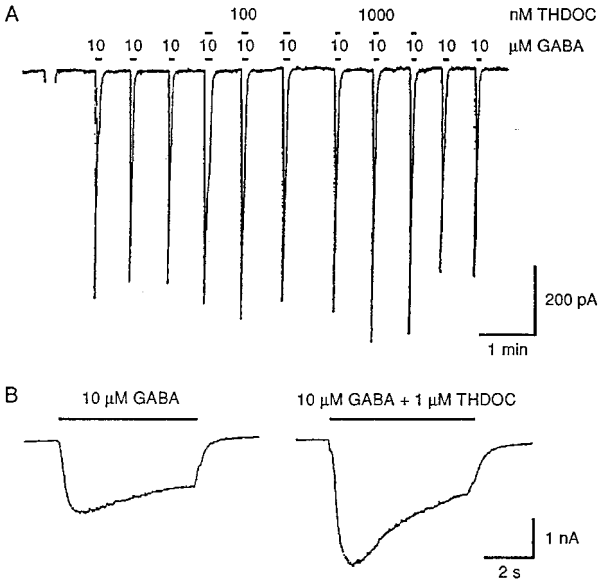

Figure 3 Enhancing effect of THDOC on Cl^- currents induced by 10 μM GABA. Simultaneous application of 10 μM GABA with 100 nM or 1 μM THDOC increased the amplitude of Cl^- currents with respect to the control. (B) Response of the neuron in (A) with different time scale.

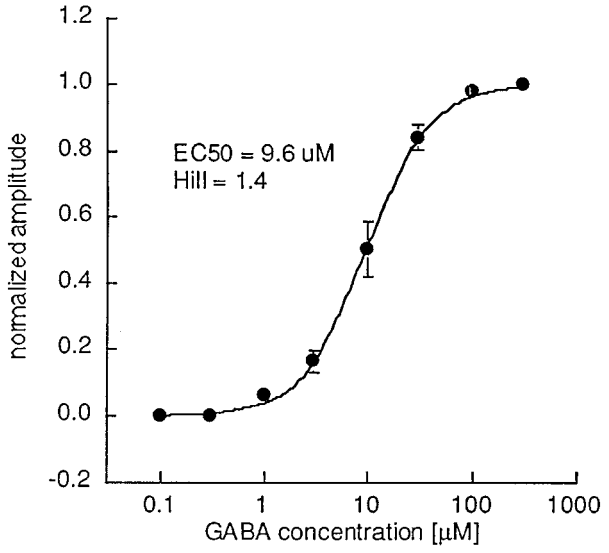

Figure 2 GABA dose-response relationship of cultured rat hypothalamic neurons. The GABA dose-response relationship had a typical sigmoidal shape. Responses with half-maximal amplitudes (EC_{50}) were induced with about 10 μM GABA ($n=9$ neurons).

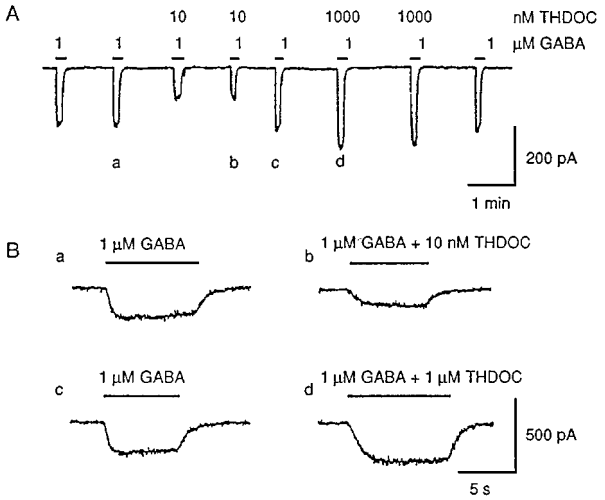

Figure 4 Modulatory effect of THDOC on Cl^- currents induced by 1 μM GABA. Simultaneous application of 10 nM THDOC with 1 μM GABA reduced the amplitude of induced Cl^- currents. Simultaneous application of 1 μM THDOC with 1 μM GABA increased the response compared to the control. (B) Current traces from the neuron shown in (A). The traces marked with (a–d) are shown with a different time scale.

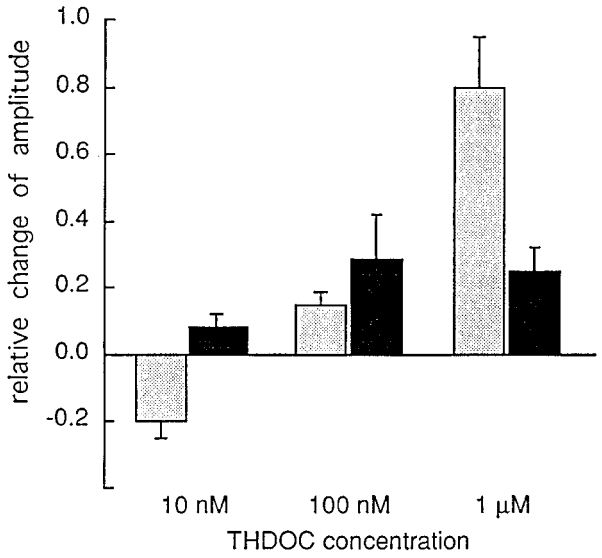

Figure 5 Average values of GABA-activated Cl^- currents of cultured rat hypothalamic neurons with and without THDOC. The amplitude of Cl^- currents induced by 10 μM GABA was increased by 100 nM and 1 μM simultaneously applied THDOC to 128% ($n=12$, $P=0.007$) and 125% ($n=17$, $P=0.005$; Wilcoxon-signed-rank test) compared to the control. 10 nM THDOC had no consistent effect on the amplitude of the GABA-induced Cl^- current ($n=9$). The amplitude of Cl^- currents evoked by 1 μM GABA were augmented by 100 nM THDOC to 115% ($n=7$, $P=0.06$, not significant) and to a much higher extent by 1 μM simultaneously applied THDOC to 180% of the control ($n=15$, $P=0.0007$). Interestingly, 10 nM THDOC decreased the amplitude of the induced Cl^- current significantly to 80% of the control ($n=15$, $P=0.018$).

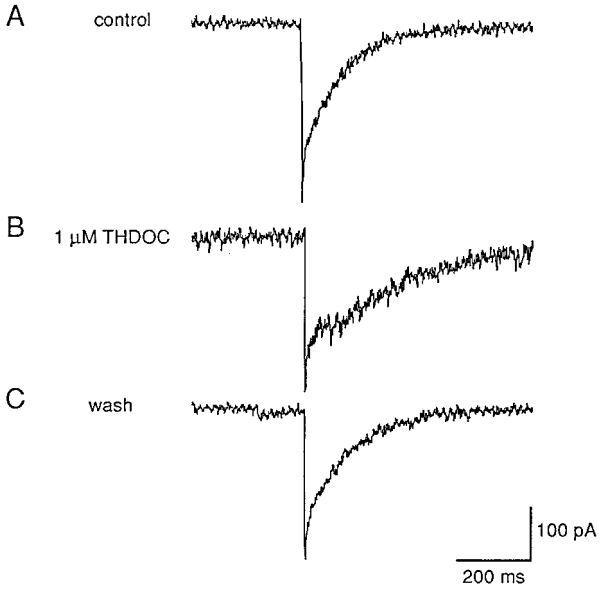

Figure 6 Effect of THDOC (1 μM) on spontaneous IPSCs. Traces of spontaneous synaptic currents in control solution (A), in the presence of 1 μM THDOC (B) and after return to control solution (C). The time constant τ_{IPSC} of the decay of the synaptic currents was prolonged about 3 fold by THDOC.

the average of three successive applications). On average, Cl^- currents induced by 10 μM GABA (see Figure 5), were augmented by 100 nM and 1 μM THDOC compared to the control, to 128% ($n=12$, $P=0.007$) and 125% ($n=17$, $P=0.005$; Wilcoxon-signed-rank test), respectively.

The responses induced by low concentrations of GABA (1 μM) were differentially affected by low concentrations of THDOC. In the neuron shown in Figure 4, 1 μM GABA induced a current of 215 pA that was enhanced by 1 μM THDOC to 280 pA, whereas application of 10 nM THDOC reduced the current to 120 pA. On average, 10 nM THDOC decreased the current induced by 1 μM GABA to 80% of control ($n=15$, $P=0.018$; see Figure 5). In these neurons, 100 nM THDOC slightly enhanced the currents induced by 1 μM GABA to 115%, but differences were not significant ($n=7$, $P=0.06$, n.s.). High concentrations of THDOC (1 μM) caused the usual enhancement of responses to GABA (1 μM) to 180% of control ($n=15$, $P=0.0007$).

THDOC-effects on spontaneous synaptic activity

To test whether these modulations have any relevance for synaptic transmission, THDOC effects on the (bicuculline-sensitive) spontaneous synaptic currents were evaluated. To mimick a background of physiological steroid level, cultures were grown in FCS-supplemented (10%) MEM. The amplitudes of the postsynaptic currents ranged from 30–290 pA and were, on average, not changed by THDOC. However, the time constant of decay of the synaptic currents (τ_{IPSC}) was increased more than 3 fold during application of THDOC (Figure 6). τ_{IPSC} was found to be 91 ± 10 ms under control conditions and 314 ± 34 ms in the presence of 1 μM THDOC ($n=3$ cells). In two neurons the washout was complete, and τ_{IPSC} recovered to the control values (92 ± 10 ms).

Discussion

Enhancement of GABA responses by THDOC

High doses of THDOC invariably augmented the neuronal responses to low GABA concentrations. The enhancing effect of 100 nM and 1 μM THDOC on 10 μM GABA-induced Cl^- currents (128 and 125% of control, respectively), however, is small compared to the effect on currents induced by 1 μM GABA (up to 180% of control). The stronger effect of THDOC on Cl^- currents induced by low GABA concentrations is remarkably similar to the effects of the progesterone metabolite tetrahydroprogesterone (THP) on the GABA-induced Cl^- currents in cultured rat hippocampal neurons: responses to 1 μM GABA are markedly enhanced by THP, but THP had little effect on the responses induced by application of 50 μM GABA (Rodgers-Neame *et al.*, 1992). The lack of effects of THP on responses to high GABA concentrations may be attributed to a saturation of the GABA response, since a maximal activation of the GABA receptor might preclude the detection of any modulation by the neurosteroid. However, the GABA concentration tested here (10 μM) GABA evokes about half-maximal responses (see Figure 2). Hence, a saturation of the GABA-induced Cl^- current as a cause of the decreased potency of THDOC appears unlikely to account for our observations. The mechanism underlying the weaker augmentation of GABA-induced currents by THDOC at about half-maximal GABA concentrations is not known and has to be clarified in future investigations.

The effect of THDOC on currents induced by 10 μM GABA did not show a distinct dose-response relationship. Low concentrations of THDOC (10 nM) had no significant effect on the current amplitudes induced by 10 μM GABA, while moderate and high concentrations of THDOC (100 nM and 1 μM) enhanced the GABA response to the same extent. This might be attributable to a steeper slope of the dose-response curve, showing no effect with 10 nM THDOC and being already saturated with 100 nM THDOC.

Bidirectional modulatory effects of THDOC on GABA responses

The bidirectional modulation of GABA currents (induced by 1 μM GABA) by different concentrations of THDOC shown here, is a new physiological feature of THDOC. Bidirectional effects were shown for the action of the neurosteroids 3α-OH-DHP (allopregnanolone) on recombinant $\alpha6\beta2\gamma2$ $GABA_A$ receptors and pregnenolone sulphate (PS) on the $GABA_A$-receptor complex in the brain (Majewska & Schwartz, 1987; Zaman *et al*., 1992; Hauser *et al*., 1995). 3α-OH-DHP and PS in the lower nanomolar range increased GABA-induced Cl^- currents, but had a GABA antagonistic effect in the upper nanomolar and micromolar range. Such a bimodal effect may be explained by two separate mechanisms with different dose-dependencies and opposite directions. At low THDOC concentrations binding of THDOC to the high affinity binding site at the $GABA_A$ receptor might reduce the amplitude of the GABA-induced Cl^- currents, whereas occupation of the low affinity binding site at higher concentrations of THDOC would increase the amplitude of the GABA-induced Cl^- current. This scheme may be the physiological consequence of multiple binding sites with different affinities for neuroactive steroids at the $GABA_A$ receptor (McCauley & Gee, 1995).

This bimodal action of THDOC on the response to application of low GABA concentrations (1 μM) may be of particular physiological relevance, as the residual GABA concentration in the synaptic cleft was found to be in the same range (Brown & Galvan, 1977). As the postsynaptically located $GABA_A$ receptors may serve as a target of steroid action, the bimodal action of THDOC may alter the signal to noise ratio of inhibitory GABAergic synaptic transmission. Depending on the THDOC concentration, the tonic background activity caused by lingering GABA in the synaptic cleft may be reduced by low and enhanced by high concentrations of THDOC. Moreover, the inhibitory postsynaptic events caused by transient high levels of GABA may be enhanced by high THDOC concentrations. This mechanism is of particular interest in brain regions such as the suprachiasmatic nuclei, where a circadian rhythm in basic GABA levels (Aguilar-Roblero *et al*., 1993) and efficacy of GABAergic transmission was found (Trachsel *et al*., 1996).

Modulation of spontaneous IPSCs by THDOC

The time constant of decay of spontaneous IPSCs (τ_{IPSC}) was increased 3 fold by THDOC. This marked prolongation of the synaptic current causes an increase of the current-time integral and therefore probably has severe consequences on the efficacy of the synaptic inhibition. A similar effect has been shown for the GABA agonistic effect of the steroid 5β-THP (epipregnanolone) in cultured rat hippocampal neurons (Harrison *et al*., 1987b), the barbiturate pentobarbitone, the anaesthetics ketamine and halothane in CA1 pyramidal cells in rat hippocampus (Gage & Robertson, 1985), and of the anticonvulsant diphenylhydantoin in the crayfish stretch receptor (Deisz & Lux, 1977). The prolongation and enhancement of GABAergic inhibitory postsynaptic events may be the common denominator for the diverse effects of neuroactive steroids, including the sedative, anaesthetic and anticonvulsant properties (Bäckström *et al*., 1990; Belelli *et al*., 1990; Deutsch *et al*., 1992; Majewska, 1992).

The prolongation of the GABA receptor-mediated IPSCs may be attributable to an increase in mean channel open life time and burst duration, as well as to an increase in the frequencies of these single-channel events (Harrison *et al*., 1987b; Lambert & Peters, 1989; Eichten & Twyman, 1994). Each of the alterations in gating would tend to increase the amplitude of macroscopic GABA-induced Cl^- currents by THDOC. During synaptic transmission, the GABA concentration in the synaptic cleft might reach saturating levels, preventing a further increase in current amplitude by the action of THDOC (compare Rodgers-Neame *et al*., 1992).

Discrepancies between THDOC effects on IPSCs and GABA responses

THDOC appears to exert two divergent effects on GABA responses and on IPSCs, the former being augmented in amplitude the latter being increased in duration. A unifying hypothesis to account for both effects may be derived from the time course of desensitization of GABA-induced currents, strongly depending upon the dose and the amplitude (see Figure 1). Near maximal responses display an approximately 3 fold shorter decay time constant, similar to GABA response in hippocampal neurons (Oh & Dichter, 1992). The accelerated decay due to augmented desensitization might counteract an increase in duration of the GABA response by THDOC at intermediate GABA concentrations, yielding an increase in amplitude without significant prolongation of the response. Only at maximal responses, when desensitization has also attained a maximum the underlying prolongation is revealed. The well balanced interplay between THDOC-induced increase in GABA current and the concomitant enhancement of desensitization provides an interesting feature: a selective increase in amplitude without changes in the kinetic of the GABA response.

In conclusion the present experiments provide evidence that the neuroactive steroid THDOC modulates the amplitudes of $GABA_A$ receptor-mediated Cl^- currents of hypothalamic neurons in a bidirectional manner. Depending on the THDOC and GABA concentration the interaction of THDOC with the $GABA_A$ receptor leads to a reduction or an augmentation of the excitability of neurons which might be involved in controlling the activity of the neuroendocrine system.

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